Proceedings of 15th Agricultural Research Symposium (2016) 135-139

Molecular Characterization of a Traditional Rice Variety *Kaluheenati* (*Oryza sativa* L.) using Simple Sequence Repeats Markers

S.M.T.T. SIRIWARDHANA[1], W.L.G. SAMARASINGHE[2], and D.R. GIMHANI[1]

[1]*Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), 60170, Sri Lanka*

[2]*Plant Genetic Resource Centre, Gannoruwa, Peradeniya, 20400, Sri Lanka*

ABSTRACT

Molecular characterization of genotypes provide exact information about the degree of genetic diversity. Assessing genetic diversity of the traditional rice variety *Kaluheenati* which has 39 accessions collected at the Plant Genetic Resource Centre is a necessity. Main objective of this study was to select a representative set of *Kaluheenati* using molecular analysis to be used in evaluation and multiplication for commercial cultivation. Three accessions were excluded from the research due to morphological incompatibility and one accession excluded due to poor germination. Total 36 accessions (35 *Kaluheenati* accessions and pure-line of *Kaluheenati* collected from Rice Research and Development Institute, Batalagoda) were evaluated using 31 Simple Sequence Repeats markers dispersed broadly over the rice genome. The DNA was extracted from tender leaves using CTAB method and PCR amplicons were separated using 8% Polyacrylamide gel electrophoresis. A significant degree of genetic diversity was found among the accessions and also heterogeneity was shown within some accessions. A total no of 113 alleles were detected and all the primers were polymorphic. Allele richness were ranged from 2 to 6 per locus, polymorphic information content values varied from 0.0094 to 0.6601. Nine closely related accessions (4089, 5191, 5385, 7802, 4087, 6713, 12932, 12926, and 4091) were selected as representative set in compare to the pure-line of *Kaluheenati*.

KEYWORDS: Genetic diversity, *Kaluheenati*, Representative set, Simple sequence repeats

INTRODUCTION

Rice (*Oryza sativa* L.) is considered as the staple food for more than half of the world's population and one of the most important cereal crop (Jiang, *et al.*, 2013). Sri Lankan rice varieties belong to indica subspecies. Paddy cultivation occupied about 964,268 hectares in year 2014 and the total production was about 3.4 million metric tons (Department of Agriculture, 2015). Presently most of the rice lands in the country are cultivated with Newly Improved Varieties (NIVs) and sometimes some traditional and old improved varieties are cultivated at minor extent (Rajapakse *et al.*, 2000). Such type of traditional varieties are *Suwadel, Kuruluthuda, Kaluheenati, Rathel, Ma-Wee, Pachchaperumal, Madathawalu,* and *Heenati etc.*

Currently, out of the total germplasm collection in the Plant Genetic Resource Centre (PGRC) Gene Bank, 4541 accessions (Annual Performance Report, 2012) are belonged to rice and related spp., including both newly improved varieties and traditional varieties. A traditional variety *Kaluheenati* (red-rice pericarp) was used in this research. It is known as highly nutritious with high fiber content which helps to regulate bowel movement. It is also known as effective in keeping diabetes under control as well as controlling the toxic effect of snake bites (Wijesena, 2014).

There are 39 accessions of *Kaluheenati* at PGRC, which have been collected from all over the country. Hence it may consist diverse array among these accessions. Current study aimed for genetic characterization of these accessions to find out the presence of duplications and other important information. Microsatellites or Simple Sequence Repeats (SSR) markers which feature high reproducibility and accuracy, were used to characterize the germplasm collection (Molin *et al.*, 2013).

MATERIALS AND METHODS

Experimental Site

The present study was carried out at the Division of Biotechnology, Plant Genetic Resource Centre (PGRC), Gannoruwa from December 2015 to May 2016.

Plant Materials

Seeds of 39 *Kaluheenati* accessions (Acc. No. 2087, 2089, 2090, 2100, 2197, 3851, 3989, 4086, 4087, 2101, 4088, 4089, 4090, 4091, 4253, 4536, 4620, 4621, 4740, 4991, 5191, 5385, 5484, 5485, 6232, 6713, 7802, 10480, 10687, 10755, 11194, 11377, 11380, 11672, 12926, 12932, 12068, 12056, 12065) were obtained from the gene bank of PGRC while pure-line of *Kaluheenati* (Acc. No. 721) was obtained from the Rice Research and Development Institute (RRDI), Batalagoda.

Extraction of Genomic DNA

Genomic DNA was extracted from two weeks old immature tender leaves using the CTAB method (Doyle and Doyle, 1990) with some modifications. Around 0.2 g of fresh leaf samples were ground with liquid N_2 using pre-cooled motor and pestle until a fine powder was formed. The contents were transferred into micro centrifuge tubes with 600 µL of pre-heated 2% CTAB extraction buffer [2% CTAB, 0.1 M Tris-HCl (pH 8.0), 0.02 M EDTA and 1.4 M NaCl]. The tubes were incubated at 65 °C in a water bath for 30 min with gentle mixing by manually at 10 min interval. After incubation, an equal amount of Chloroform:Isoamyl alcohol (24:1) was added to each tube and slowly shaken for 20 min at room temperature on a rotary platform for better emulsification of two phases. Then, all the samples were centrifuged at 10,000 rpm for 15 min

The supernatant was transferred into new micro centrifuge tube without disturbing the interface. Nearly $1/10^{th}$ of 10% CTAB was added into each tube and equal volume of chloroform:Isoamyl alcohol (24:1) was added again to the same tube and shaken well for 20 min for purification of DNA from protein. Samples were centrifuged at 10,000 rpm for 15 min Again, the supernatant was transferred into another micro centrifuge tube and $2/3^{rd}$ volume of ice-cold Isopropanol was added and mixed gently by inverting. Whitish thread like DNA was spooled out.

The spooled DNA samples were washed two times with 70% ethanol by centrifuging at 10,000 rpm for 5 min Ethanol was carefully drained out and the pellet was air dried. Finally, DNA pellet was resuspended in TE buffer [0.01 M Tris-HCl, 0.001 M EDTA (pH 8.0)] according to the size of the pellet and allowed to dissolve it overnight at room temperature. After fully dissolution, DNA was stored at -20 °C. Extracted DNA was diluted to 15 ng/µL to be used in PCR.

Selection of Primers

Thirty one SSR markers dispersed broadly over the 12 rice chromosomes were selected and primer sequences were obtained from the published sequence database of Gramene (www.gramene.org; Table 1).

Preparation of DNA Bulks

DNA bulks were prepared based on the previous morphological characterization of seeds of 39 *Kaluheenati* accessions (Figure 1; unpublished data). Accordingly, eight DNA bulks were prepared at $0.00<x<6.58$ distance level (Figure 1; Table 2). Three accessions (Acc. No. 2089, 2100, and 5484) were excluded from this study due to morphological incompatibility identified based on the rice catalog on properties of traditional rice varieties (Industrial Technology Institute and Department of Agriculture, 2008) and one accession (Acc. No. 10755) was excluded due to poor germination.

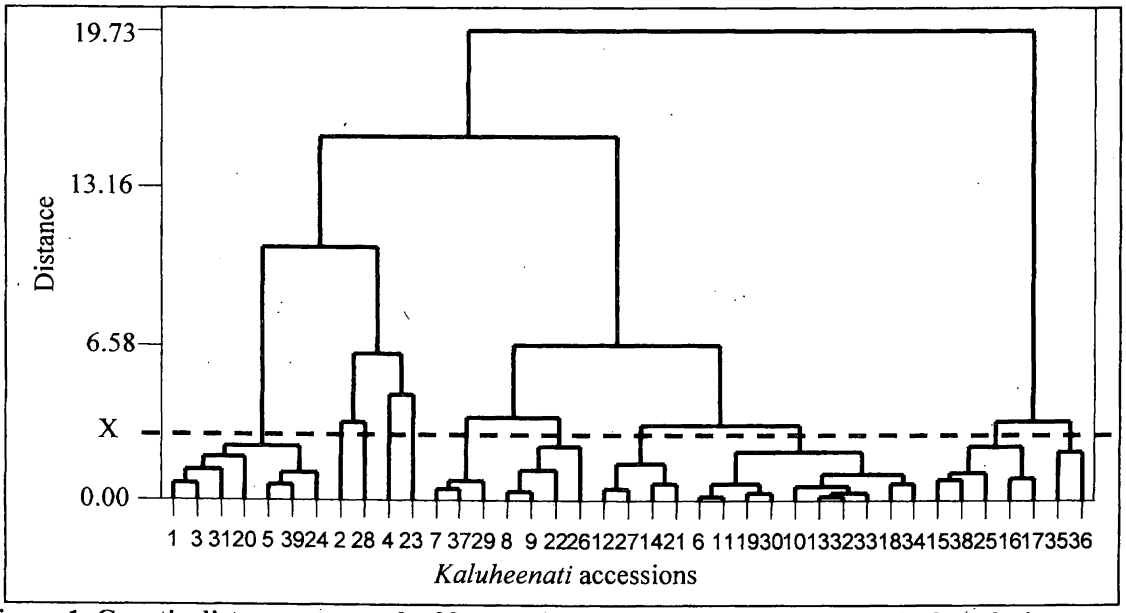

Figure 1. Genetic distance among the 39 accessions based on seed morphological analysis (Unpublished data). *1-2087, 3-2090, 31-11194, 20-4991, 5-2197, 39-12065, 24-5485, 2-2089, 28-10480, 4-2100, 23-5484, 7-3989, 37-12068, 29-10687, 8-4086, 9-4087, 22-5385, 26-6713, 12-4089, 27-7802, 14-4091, 21-5191, 6-3851, 11-4088, 19-4740, 30-10755, 10-2101, 13-4090, 32-11377, 33-11380, 18-4621, 34-11672, 15-4253, 38-12056, 25-6232, 16-4536, 17-4620, 35-12926, 36-12932*

Table 1. Sequences and annealing temperatures of selected 31 Simple Sequence Repeat (SSR) markers

Primer name	Forward sequence and reverse sequence (5'-3')	Annealing temperature (°C)	Primer name	Forward sequence and reverse sequence (5'-3')	Annealing temperature (°C)
RM20B	F-atcttgtccctgcaggtcat R-gaaacagaggcacatttcattg	55	RM236	F-gcgctggtggaaaatgag R-ggcatccctctttgattcctc	55
RM25	F-ggaaagaatgatcttttcatgg R-ctaccatcaaaaccaatgttc	55	RM237	F-caaatcccgactgctgtcc R-tgggaagagagcactacagc	58
RM84	F-taagggtccatccacaagatg R-ttgcaaatgcagctagagtac	55	RM241	F-gagccaaataagatcgctga R-tgcaagcagcagatttagtg	55
RM201	F-ctcgtttattacctacagtacc R-ctacctcctttctagaccgata	55	RM255	F-tgttgcgtgtggagatgtg R-cgaaaccgctcagttcaac	55
RM202	F-cagattggagatgaagtcctcc R-ccagcaagcatgtcaatgta	55	RM259	F-tggagtttgagaggaggg R-cttgttgcatggtgccatgt	55
RM207	F-ccattcgtgagaagatctga R-cacctcatcctcgtaacgcc	56	RM270	F-ggccgttggttctaaaatc R-tgcgcagtatcatcggcgag	55
RM208	F-tctgcaagccttgtctgatg R-taagtcgatcattgtgtggacc	55	RM412	F-cacttgagaaagttagtgcagc R-cccaaacacacccaaatac	55
RM213	F-atctgtttgcaggggacaag R-aggtctagacgatgtcgtga	55	RM418	F-tcgcgtatcgtcatgcatag R-gagcacatatgccacgtacg	55
RM215	F-caaaatggagcagcaagagc R-tgagcacctccttctctgtag	55	RM440	F-catgcaacaacgtcaccttc R-atggttggtaggcaccaaag	55
RM216	F-gcatggccgatggtaaag R-tgtataaaaaccacacggcca	55	RM480	F-gctcaagcattctgcagttg R-gcgcttctgcttattggaag	57
RM217	F-atcgcagcaatgcctcgt R-gggtgtgaacaaagacac	55	RM515	F-taggacgaccaaagggtgag R-tggcctgctctctctctctc	55
RM219	F-cgtcggatgatgtaaagcct R-catatcggcattcgcctg	56.5	RM518	F-ctcttcactcactcaccatgg R-atccatctggagcaagcaac	55
RM220	F-ggaaggtaactgtttccaac R-gaaatgcttcccacatgtct	55	RM536	F-tctctcctcttgtttggctc R-acacaccaacacgaccacac	55
RM224	F-atcgatcgatcttcacgagg R-tgctataaaaggcattcggg	55	RM539	F-gagcgtccttgttaaaaccg R-agtagggtatcacgcatccg	55
RM228	F-ctggccattagtccttgg R-gcttgcggctctgcttac	55	RM560	F-gcaggaggaacagaatcagc R-agcccgtgatacggtgatag	55
			RM571	F-ggaggtgaaagcgaatcatg R-cctgctgctctttcatcagc	55

Table 2. The DNA Bulks of *Kaluheenati*

Bulks	Accession numbers
1st Bulk	2087, 2090, 11194, 4991, 2197, 12065, 5485
2nd Bulk	10480
3rd Bulk	3989, 12068,,10687
4th Bulk	4086, 4087, 5385, 6713
5th Bulk	4089, 7802, 4091, 5191
6th Bulk	3851, 4088, 4740, 2101, 4090, 11377, 11380, 4621,11672
7th Bulk	4253, 12056, 6232, 4536, 4620
8th Bulk	12926, 12932

Polymerase Chain Reaction

All the DNA Bulks were amplified using the selected 31 SSR primers. The PCR reaction was performed in 10 µL of PCR mixture containing of 3 ng/µLof template DNA, 1X PCR buffer (Promega, USA), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM forward and reverse primers (Integrated DNA Technologies, USA), 0.4 U Taq DNA polymerase (Promega, USA). Final volume of the mixture was adjusted using sterile distilled water. PCR amplification was performed using Applied Biosystems thermo cycler (model No. 9902) and amplification profile consisted of five touchdown cycles in which the annealing temperature is reduced by 3 °C at each cycle (Table 3). Amplified products were confirmed by 1.5% agarose gel.

Table 3. The PCR programme for 55 °C annealing temperature primers

Initial denaturation - 94 °C – 4 min		
Denaturation – 95 °C – 1 min		
Annealing	**Extension**	**Cycles**
70 °C – 1 min	72 °C – 1 min	1
67 °C – 1 min	72 °C – 1 min	1
64 °C – 1 min	72 °C – 1 min	1
61 °C – 1 min	72 °C – 1 min	1
58 °C – 1 min	72 °C – 1 min	1
55 °C – 1 min	72 °C – 1 min	30
Final extension – 72 °C – 5 min		
Soaking temperature – 4 °C		

Analysis of PCR Products using PAGE

PCR products were analyzed by 8% non-denaturing PAGE. Some bulks which showed more than one allele were expanded to identify the heterogeneity within the bulks.

Data Scoring and Analyzing

Gel images were scored manually by visual observations. Allelic data of SSR markers were analyzed and cluster analysis was performed according to the Unweighted Pair Group Method with Arithmetic Mean

(UPGMA) with the Nei *et al.* (1983) similarity index using PowerMarker 3.25 version (Liu and Muse, 2005).

RESULTS AND DISCUSSION

Present results revealed the considerable level of genetic diversity among the *Kaluheenati* accessions. Accordingly, the total number of alleles detected were 113 ranging from 2-6 alleles (mean 3.65) per locus (Table 4). The highest polymorphism observed with the RM515 and RM25 SSR markers on chromosome 8 yielding 6 alleles and the lowest polymorphism was observed with RM236 and RM208 on chromosome 2, RM215 on chromosome 9, RM560 on chromosome 7, and RM270 on chromosome 12 yielding 2 alleles per locus (Table 4). The Polymorphic Information Content (PIC) value, which measures the allelic diversity at a locus, ranging from 0.0994 (RM270) to 0.6601 (RM418) (Table 4). Almost all the selected SSR markers were associated with the comparatively higher PIC values indicating informativeness of these SSR markers, which could be utilized in future genetic diversity assessments of the rice germplasm.

Out of 36 accessions including *Kaluheenati* pure-line, 20 accessions were heterogeneous for different loci. Although, rice is a highly self-pollinated crop, in some cases variations could be detected due to cross-pollination which may occur in some instances due to temperature stress and some characteristics of floral organs (Matsui and Kagata, 2003). In addition, heterogeneity within the accessions could also be resulted due to mechanical mixing of seed samples.

According to the resulted dendogram, the lowest genetic distance (0.00) was observed between accessions of 12065/2197, 5485/2090, 11380/11672 and 12926/12932 (Figure 2). Therefore, these accessions can be considered as duplicates. According to the morphological analysis also these pair of accessions clustered together indicating the duplication of these accessions (Figure 1). Pure-line of *Kaluheenati* (Acc. No. 721) showed lowest genetic distance with accession 4089 (0.468) and highest distance with 10480 (0.839). Visual appearance of the seed characteristics also showed the similarities in pure-line of *Kaluheenati* and 4089 accession. Moreover, according to the seed morphological analysis, 10480 accession was bulked with excluded accessions (Acc. No. 2089, 2100, and 5484) at 6.58 distance proving the distant genetic relationship with the pure-line (Acc. No. 721) of *Kaluheenati* (Figure 1).

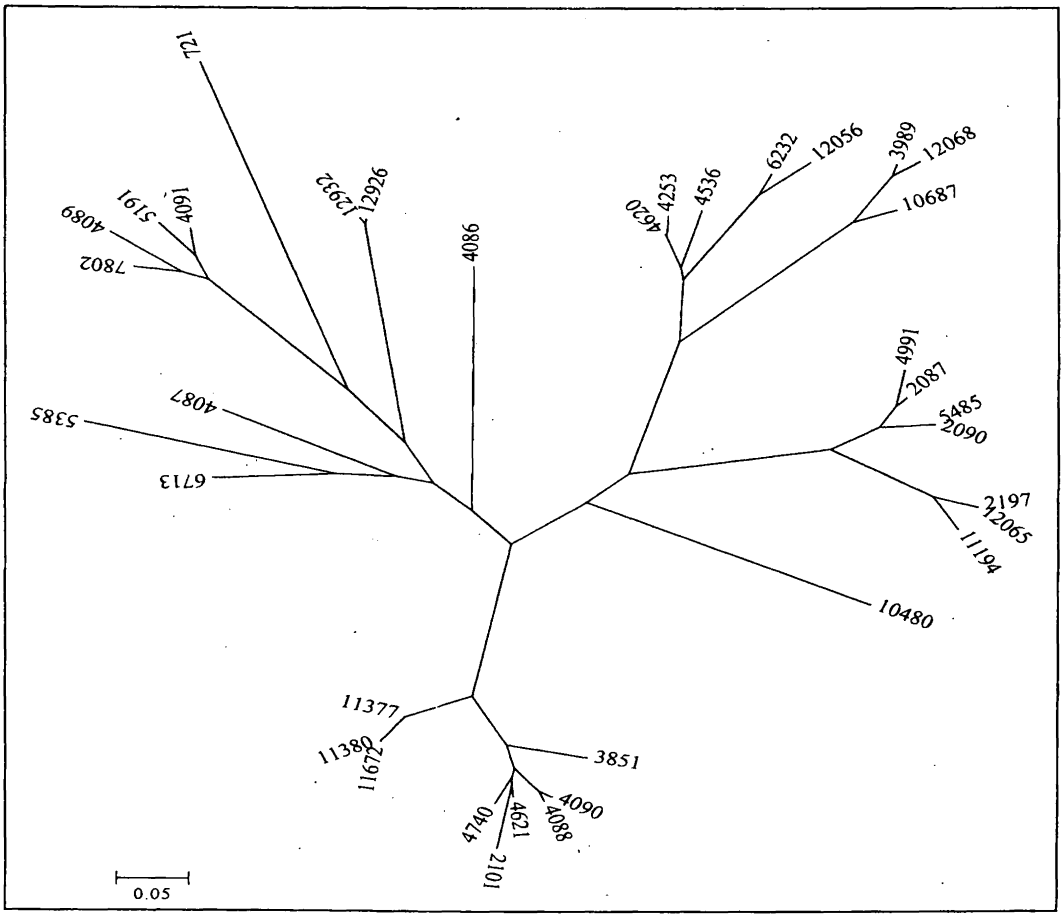

Figure 2. Dendogram of *Kaluheenati* accessions based on analysis of Simple Sequence Repeat (SSR) data

Table 4. Analysis results of primers

Marker	Allele No	Polymorphic information content	Marker	Allele No	Polymorphic information content
RM220	4	0.5989	RM25	6	0.6447
RM412	3	0.2085	RM201	4	0.6250
RM571	3	0.5308	RM216	3	0.4183
RM236	2	0.2859	RM440	5	0.5799
RM539	5	0.5572	RM228	3	0.5009
RM208	2	0.1780	RM270	2	0.0994
RM84	3	0.5349	RM213	3	0.3308
RM255	3	0.3504	RM518	4	0.6069
RM20B	3	0.5579	RM480	3	0.3709
RM259	4	0.4843	RM237	4	0.4060
RM241	5	0.6091	RM207	4	0.4376
RM515	6	0.5738	RM219	3	0.4101
RM418	5	0.6601	RM202	5	0.6479
RM215	2	0.3047	RM536	3	0.5269
RM217	4	0.5928	RM224	5	0.5552
RM560	2	0.3680	Mean	3.65	0.4695

In addition to the 4089 accession, another eight accessions (Acc. No. 5191, 5385, 7802, 4087, 6713, 12932, 12926, and 4091) also exhibited minimum distance with pure-line of *Kaluheenati* (Acc. No. 721) indicating their closeness to the *Kaluheenati* pure-line (Figure 2). Furthermore, accession 6713, which was received from International Rice Research Institute (IRRI), Philippines (Plant Genetic Resource Centre, 1999) was clustered with *Kaluheenati* pure-line, indicating the accuracy of the representative sample stored in IRRI.

CONCLUSIONS

In the present study, a significant degree of genetic diversity was found among the *Kaluheenati* 39 accessions stored at PGRC. Some accessions were heterogeneous. Nine accessions *viz.*, 4089, 5191, 5385, 7802, 4087, 6713, 12932, 12926, and 4091 were found with close relatedness to pure-line of *Kaluheenati*. Accessions 12065/2197, 5485/ 2090, 11380 /11672, and 12926/ 12932 were can be considered as duplicates, as they showed lowest genetic distances between them.

ACKNOWLEDGEMENTS

The authors offer their sincere thanks to all the staff members of the Division of Biotechnology, PGRC, and Dr. S.K. Wasala for the help given in statistical analysis.

REFERENCES

Annual performance report (2012). Department of Agriculture. Peradeniya, Sri Lanka.

Department of Agriculture. (2015). Agstat. **12**, 8, Peradeniya, Sri Lanka.

Doyle, J.J. and Doyle, J.N. (1990). Isolation of plant DNA from fresh tissue. *Focus,* **12**, 13-15.

Industrial Technology Institute and Department of Agriculture (2008). *Properties of some Traditional Rice Varieties of Sri Lanka.* Sri Lanka, Ceylon shipping Lines Ltd. Printing Department.

Jiang, S.H., Zhou, H., Lin, D.Z., Dong, Y.J., Ye, S.H. and Zhang, X.M. (2013). Identification and gene mapping of a thermo-sensitive leaf-color mutant at seedling stage in rice. *Chinese Journal of Rice Science*, **27**, 359 - 364.

Liu, K. and Muse, S.V. (2005). Power Marker: Integrated analysis environment for genetic marker data. Bioinformatics. **21**, 2128-2129.

Matsui, T. and Kagata, H. (2003). Characteristics of Floral Organs Related to Reliable Self-pollination in Rice (*Oryza sativa* L.). *Annals of Botany*, **91** (4), 473-477.

Molin, D., Coelho, C.J., Máximo, D.S., Ferreira, F.S., Gardingo, J.R. and Matiello, R.R. (2013). Genetic diversity in the germplasm of tropical maize landraces determined using molecular markers. *Genetics and Molecular Research,* **12** (1), 99-114.

Nei, M., Tajima, F. and Tateno, Y. (1983). Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. *Journal of Molecular Evaluation,* **19** (2), 153-170.

Plant Genetic Resource Centre (1999). Plant Genetic Resource Catalouge. **2**, Peradeniya, Sri Lanka.

Rajapakse, R.M.T., Sandanayake, C.A. and Pathinayake, B.D. (2000). Foot prints in rice variety improvement and its impact on rice production in Sri Lanka. Annual symposium of the Department of Agriculture, Sri Lanka, **2**, 423 - 433.

Wijesena, K.A.K. (2014). 'Ape paramparika wee prabedha'. *Govikam Sanagarawa*, **45** (3), 1-6.